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UCP2 protects hypothalamic cells from TNF-α-induced damage

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Abstract **Uncoupling protein 2 (UCP2) is highly expressed in the hypothalamus; however, little is known about the functions it exerts in this part of the brain. Here, we hypothesized that UCP2 protects hypothalamic cells from oxidative and pro-apoptotic damage generated by inflammatory stimuli. Intracerebroventricular injection of tumor necrosis factor alpha (TNF-α)-induced an increase of UCP2 expression in the hypothalamus, which was accompanied by increased expression of markers of oxidative stress and pro-apoptotic proteins. The inhibition of UCP2 expression by an antisense oligonucleotide enhanced the damaging effects of TNF-α. Conversely, increasing the hypothalamic expression of UCP2 by cold exposure reversed most of the effects of the cytokine. Thus, UCP2 acts as a protective factor against cellular damage induced by an inflammatory stimulus in the hypothalamus.**

Keywords: Inflammation; Cytokine; Apoptosis; Neuron; Hypothalamus; Obesity

1. Introduction

The uncoupling proteins (UCPs) are a family of anion-carrier proteins expressed in the inner membrane of the mitochondria [1]. In brown adipose tissue, the prototypic UCP1 drives the leakage of protons from the intermembrane space back to the mitochondrial matrix, wasting the electrochemical gradient as heat [2]. During the last 10 years, four additional UCP homologous proteins have been identified and shown to be expressed in several tissues of the body, besides adipose [1]. One of these homologues, uncoupling protein 2 (UCP2), is highly expressed in the central nervous system, predominantly in the hypothalamus, limbic system, cerebellum, choroid plexus and brainstem [3]. Although, to a certain degree, UCP2 can exert a typical uncoupling and thermogenic function [4], the main role of this protein in the brain is still under intense investigation [5]. There are a number of studies showing that UCP2 may have a role in the control of neuronal intracellular calcium, ATP production, mitochondrial biogenesis, synaptic transmission, neuronal plasticity and reactive oxygen species (ROS) production [6].

Overproduction or accumulation of ROS in neurons is regarded as an important mechanism predisposing to the development of neurodegenerative diseases [6]. The generation of an increased mitochondrial membrane potential leads to a parallel random single electron transfer reaction from components of the electron transfer chain to molecular oxygen, boosting the production of ROS [7]. Increasing the expression of UCP2 in the mitochondria of neurons mitigates ROS production, therefore protecting these cells from the damage from oxidative stress [4].

The activation of inflammatory signaling is an important mechanism leading to increased ROS production in several cell types, including neurons [6]. The pro-inflammatory cytokine, tumor necrosis factor alpha (TNF-α), is regarded as an important mediator of neuronal damage in a number of inflammatory and degenerative diseases of the central nervous system [8]. One of the mechanisms involved in TNF-α-induced neuronal damage is the overproduction of ROS [9,10].

In the present study, we tested the hypothesis that UCP2 can act as an endogenous protective factor against inflammatory-induced damage in the central nervous system. Our results show that inhibiting UCP2 expression increases TNF-α-induced expression of markers of ROS accumulation and apoptosis, while increasing UCP2 expression by cold exposure restrains the damage caused by the cytokine.

2. Materials and methods

2.1. Antibodies, chemicals and buffers

Reagents for SDS–PAGE and immunoblotting, HEPES, phenylmethylsulfonyl fluoride, aprotinin, dithiothreitol, Triton X-100, Tween 20 and glycerol, were from Bio-Rad (Richmond, CA, USA). Sodium thiopental was from Lilly (Indianapolis, IN, USA). Anti-UCP2 (sc-6525, goat polyclonal), anti-Bcl-2 (sc-492, rabbit polyclonal), anti-Bax (sc-493, rabbit polyclonal), anti-actin (sc-7210, rabbit polyclonal), anti-cytochrome-*c* (sc-13561, mouse monoclonal), and anti-superoxide dismutase 1 (SOD1) (sc-8637, goat polyclonal) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-catalase antibody (#C0979, mouse monoclonal) and hydroethidine were from Sigma (St. Louis, MO, USA).

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Abbreviations: CAT, catalase; ICV, intracerebroventricular; IP, intraperitoneal; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling; UCP2, uncoupling protein 2

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2.2. Experimental animals

Male Wistar rats of 8–10 weeks (200–250 g) from the State University of Campinas Breeding Center, were used in the study. The investigation followed the University guidelines for the use of animals in experimental studies and conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The animals were maintained on a 12:12 h artificial light:dark cycle and housed in individual cages. Standard rodent chow and water were provided ad libitum.

2.3. Sense and antisense oligonucleotide treatment protocols

Sense and antisense oligonucleotides (produced by Invitrogen Corp., Carlsbad, CA, USA) were diluted to a final concentration of 10 nmol/ml in dilution buffer containing 10 mmol/l Tris–HCl and 1.0 mmol/l EDTA. The rats were injected intraperitoneal (IP) with one daily dose of 200 μl of dilution buffer containing, or not, sense (UCP2so) or antisense oligonucleotides (UCP2aso). The oligonucleotides were designed according to the UCP2 sequence deposited at the NIH-NCBI (http://www.ncbi.nlm.nih.gov/entrez) under the designation NM 011671 and were composed of 5′-TGC ATT GCA GAT CTC A-3′ (sense) and 5′-TGA GAT CTG CAA TGC A-3′ (antisense). The effectiveness of the oligonucleotides to inhibit UCP2 expression, has been previously shown [11]. The treatment with the sense oligonucleotide produced no difference in the expression of UCP2 as compared to control (data not shown).

2.4. Intracerebroventricular (ICV) cannulation

Male Wistar rats were stereotaxically instrumented to receive a cannula placed in the lateral ventricle, as previously described [12]. After 7 days, the correct location of the cannula was tested by injecting 2.0 μl angiotensin II (10^{-6} M) and determining the thirst response [13].

2.5. TNF-α and cold exposure protocols

In a series of experiments, male Wistar rats were either maintained at room temperature (22 °C) or exposed to cold (4 °C) for 9 days [14]; from day 3 on, groups at room temperature were sub-divided and treated ICV, at 9 a.m., either with saline or TNF-α (10^{-12} M, 2.0 μl); at the end of the experimental period (day 9) the animals were killed at 9 a.m. by deep anesthesia and the hypothalami prepared for further analysis. This experiment was carried out five times. For another series of four consecutive repeated experiments, age-matched male Wistar rats were maintained at room temperature (22 °C) and treated for 9 days with a single IP, daily dose of UCP2aso or dilution buffer; from day 3 on, each group was sub-divided and treated ICV at 9 a.m. either with saline or TNF-α (10^{-12} M, 2.0 μl); at the end of the experimental period (day 9) the animals were killed at 9 a.m. by deep anesthesia and the hypothalami were prepared for analysis. Finally, in a series of four consecutive repeated experiments, age-matched male Wistar rats were either maintained at room temperature (22 °C) or in a cold room (4 °C) and treated for 9 days with a single IP, daily dose of UCP2aso or dilution buffer. All groups were subjected to one single ICV injection of TNF-α (10^{-12} M, 2.0 μl), 24 h before hypothalamus preparation.

2.6. Hypothalamic specimen preparation for terminal deoxynucleotidyl-transferase-mediated dUTP nick end-labeling (TUNEL)

Rats were submitted to deep anesthesia, the chest was opened, a needle was inserted into the left ventricle, and the descending aorta was opened. Whole body perfusion involved sequential injections of 0.9% saline solution (until the blood was washed out), followed by 4% paraformaldehyde for 30 min. The rats were then decapitated; the whole brain was removed, and stored overnight at 4 °C in a vial containing 4% paraformaldehyde. Coronal sections were carried out beginning anterior to the optic chiasm and spanning the hypothalamus up to the region posterior to the mammillary peduncle. The portion containing the hypothalamus was paraffin-embedded and manually sectioned (5.0 μm) using a Leica RM2125 microtome (Wetzlar, Germany). Sections were placed on Vectabond reagent processed glass slides (Vector Laboratories, Burlingame, CA, USA) and air-dried overnight at room temperature. Sections were deparaffinized in xylene (three washes), followed by rehydration in decreasing concentrations (100%, 95% and 70%) of ethanol.

2.7. Detection of apoptosis of hypothalamic cells by TUNEL

A TUNEL assay was used to identify double-stranded DNA fragmentation. Briefly, tissue slides after deparaffinization, were treated with proteinase K (20 μg/ml) for 15 min at room temperature, and then quenched in 2.0% hydrogen peroxide. After rinsing in phosphate-buffered saline (PBS), pH 7.4, specimens were incubated in 1× equilibration buffer for 10–15 s. The slides were then incubated with terminal deoxynucleotidyl transferase (TdT) for 1 h at 37 °C, blocked with stop/wash buffer, and incubated with peroxidase antibody for 30 min at room temperature. The TUNEL assay negative control consisted of staining the tissues in the same manner without the antibody. The percentage of TUNEL-positive cells in lateral hypothalamus was determined in five non-consecutive sections obtained from each tissue block ($n = 5$).

2.8. Detection of apoptosis of hypothalamic cells by the annexin method

Rats were submitted to deep anesthesia, the chest was opened, a needle was inserted into the left ventricle, and the descending aorta was opened. Whole body perfusion involved sequential injections of 0.9% saline solution, until the blood was washed out. After decapitation, the brain was rapidly removed and deep-frozen in liquid nitrogen. Five micrometers sections were obtained from regions located between bregma 0.0 and −5.0 mm. The sections were incubated with FITC-conjugated annexin V (1:500) in a dark chamber. After washing, the slides were mounted and evaluated by fluorescence microscopy.

2.9. Detection of O^{2-} and O^{2-} derived oxidant production

Two hundred microliters of PBS containing 1.0 μg/μl hydroethidine were injected IP and after 15 min the hypothalami were harvested, frozen on dry ice and sectioned in 14 μm thick sections. After mounting in gelatin-coated slides, ethidium accumulation was evaluated by fluorescence microscopy (excitation, 510 nm; emission 580 nm). Results are presented as direct comparison between groups. Detailed description of the method was originally published by Wu et al. [15].

2.10. Mitochondria preparation for use in immunoblot experiments

Hypothalamic mitochondria were isolated by homogenization in ice-cold medium containing 100 mmol/l sucrose, 100 mmol/l KCl, 50 mmol/l Tris–HCl, 1.0 mmol/l K_2HPO_4, 0.1 mmol/l EGTA, and 0.2% BSA, pH 7.4, followed by differential centrifugation, as described [16]. Samples containing 80 μg mitochondria protein were separated by SDS–PAGE, transferred to nitrocellulose membranes, and blotted against UCP2, Bax, Bcl-2 and cytochrome-*c* antibodies. The quality of the mitochondria preparation was evaluated by the determination of in vitro respiration, as previously described [16].

2.11. Immunoblotting

Hypothalami were excised and immediately homogenized in solubilization buffer at 4 °C [1% Triton X-100, 100 mmol/l Tris–HCl (pH 7.4), 100 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l EDTA, 10 mmol/l sodium orthovanadate, 2.0 mmol/l PMSF and 0.1 mg aprotinin/ml] with a Polytron PTA 20S generator (model PT 10/35; Brinkmann Instruments, Westbury, NY, USA). Insoluble material was removed by centrifugation for 40 min at 11 000 rpm in a 70 Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentration of the supernatants was determined by the Bradford dye binding method. In direct immunoblot experiments, 80 μg of protein extracts obtained from hypothalamus were separated by SDS–PAGE, transferred to nitrocellulose membranes, and blotted with antibodies against UCP2, catalase (CAT), superoxide dismutase (SOD) and actin.

2.12. Immunohistochemistry

Paraformaldehyde-fixed hypothalami were sectioned (5.0 μm) and used in regular single-immunofluorescence staining using UCP2 antibodies as previously described [11]. Analysis and documentation of results were performed using a Leica FW 4500 B microscope (Wetzlar, Germany). The hypothalami were sectioned from Bregma −1.8 to −3.6.

2.13. Statistical analysis

Specific protein bands present in the blots were quantified by digital densitometry (ScionCorp Inc., Frederick, MD, USA). Mean values ± S.E. obtained from densitometric scans, and numeric data from

TUNEL and mitochondria respiration assays were compared utilizing ANOVA with Bonferroni post-test and *t*-test. A P value of <0.05 was accepted as statistically significant.

3. Results

3.1. TNF-α induces apoptosis in hypothalamus

The ability of TNF-α to induce apoptosis in the hypothalamus was studied by means of two different methods. First, a group of five rats were treated for 6 days with a single dose (2.0 μl, 10^{-12} M) of TNF-α and, at the end of the experimental period, the hypothalami were obtained and used for analysis by the TUNEL method. As shown in Fig. 1A, TNF-α promoted a 2.2-fold increase in TUNEL-positive cells. Next, another group of five rats were treated, according to the same protocol as above, and at the end of the experimental period, freshly removed specimens were evaluated by the annexin V method. The semi-quantitative evaluation revealed a significant difference in annexin V-positive cells between control and TNF-α-treated rats, as shown in Fig. 1B.

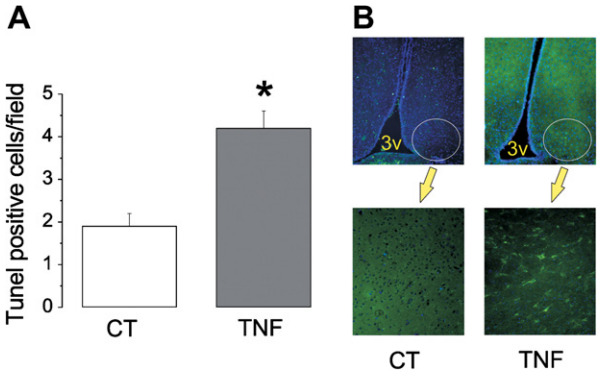

Fig. 1. Detection of apoptosis of hypothalamic cells. (A) Analysis of TUNEL-positive staining (cells/field) of animals treated either with TNF-α (10^{-12} M) or saline (CT). Values are means ± S.E.M., of five distinct observations ($^{*}P < 0.05$ vs. CT). (B) Representative annexin stained sections of rat hypothalamus. Upper panels, low magnification (50×), depicting third ventricle (3v) and surrounding nuclei at Bregma −2.6. Lower panels, high magnification (200×) depicting the arcuate nucleus. Rats were treated ICV either with TNF-α or saline (CT) ($n = 5$).

3.2. Modulation of UCP2 expression in hypothalamus

Initially, the quality of the mitochondria preparation was evaluated by determining the effects of TNF-α and cold exposure on the respiration pattern of isolated mitochondria. As depicted in Fig. 2A, both the ICV TNF-α treatment and the exposure of experimental animals to +4 °C led to a significant fall in the respiratory rates of the isolated mitochondria, a finding that is in accordance with previous studies [17]. Next, we determined the capacity of detecting UCP2 expression by the immunoblot technique. As shown in Fig. 2B, UCP2 could be easily detected in isolated mitochondria from hypothalamus and white adipose tissue, but not from the heart of rats. Using whole tissue extracts, the sensibility of the method was much lower (Fig. 2B, right-hand side panel). Therefore, in the remainder of the experiments designed to evaluate UCP2 expression (and also Bax and Bcl-2 expression), only hypothalamic purified mitochondria preparations were used. To reduce UCP2 expression, rats were treated for 9 days with a single IP dose (dose response ranging from 0.0 to 2.0 nmol/day) of UCP2aso. As shown in Fig. 2C, the doses of 1.0 and 2.0 nmol/day were capable of inhibiting most of the expression of the immunoreactive UCP2 in isolated mitochondria. The effect of the antisense oligonucleotide (2.0 nmol/day) to reduce UCP2 expression was further confirmed by immunofluorescence

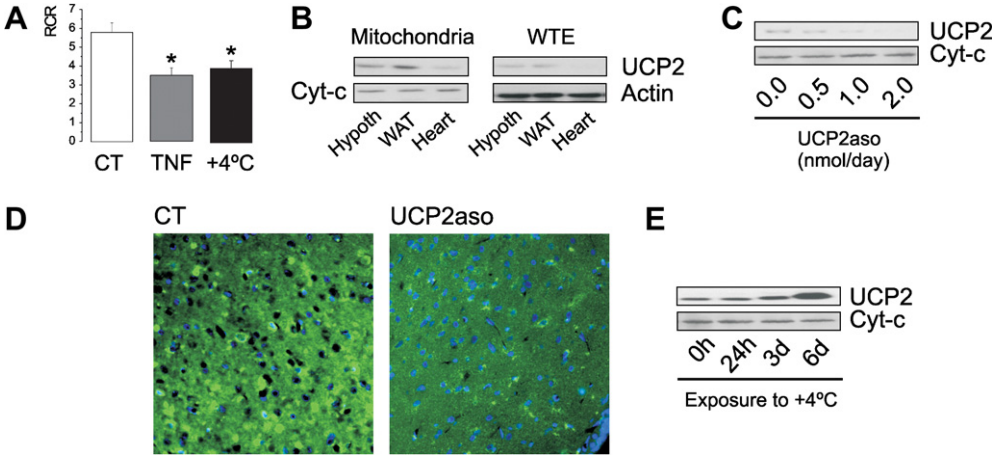

Fig. 2. Evaluation of mitochondrial preparation and UCP2 expression. (A) Respiratory rates (nmol O_2/ml) of purified mitochondria preparations of rat hypothalami treated with saline (CT), TNF-α (10^{-12} M; single ICV injection) or by cold exposure (4 °C) ($n = 3$). (B) In the left-hand side panels, UCP2 expression was analyzed by immunoblot in purified mitochondria preparations from the hypothalamus (Hypoth), the white adipose tissue (WAT) and the heart. In the right-hand side panels, UCP2 expression was analyzed by immunoblot in the whole tissue extracts (WTE) of hypothalamus (Hypoth), white adipose tissue (WAT) and heart ($n = 4$); loading controls were blotted with anti-cytochrome-*c* (Cyt-*c*) (for mitochondria) and anti-actin antibodies (for whole tissue extract). (C) UCP2 expression was analyzed in rats pre-treated (for 9 days, single IP injection/day) with UCP2 antisense oligonucleotides (UCP2aso) in the following concentrations: 0, 0.5, 1.0 and 2.0 nmol/day, respectively; samples were immunoblotted with anti-UCP2 and anti-cytochrome-*c* antibodies ($n = 4$). (D) Anti-UCP2 immunohistochemical staining of 5.0 μm sections of paraformaldehyde fixed hypothalami of control (CT) or UCP2 antisense oligonucleotide treated (2.0 nmol/day, for 9 days, single IP injection/day) rats. (E) Determination of UCP2 expression in hypothalami of rats maintained at room temperature (22 °C) or exposed to cold (4 °C) during 24 h, 3 days and 6 days, samples were immunoblotted with anti-UCP2 or anti-cytochrome-*c* antibodies ($n = 4$). Values are means ± S.E.M. ($^{*}P < 0.05$ vs. CT).

staining of the hypothalamus with the anti-UCP2 antibody (Fig. 2D). Thus, in the remainder of the experiments aimed at inhibiting the expression of UCP2, the dose of 2.0 nmol/day was always employed. Finally, to increase UCP2 expression in hypothalamus, we exposed the experimental animals to a cold environment. As shown in Fig. 2E, after three and, particularly, after 6 days of cold exposure a remarkable increment in UCP2 expression in mitochondria from hypothalamic cells was observed.

3.3. UCP2 expression inhibition increases TNF-α-induced apoptosis and ROS accumulation in the hypothalamus

In rats treated ICV with TNF-α, the expression of UCP2 in hypothalamic mitochondria was increased by approximately two-fold, as compared to the control (Fig. 3A). The use of the UCP2 antisense oligonucleotide significantly inhibited the effect of TNF-α, maintaining the levels of immunoreactive UCP2 similar to that of the control. Under UCP2 inhibition, the effects of TNF-α on markers of apoptosis were significantly magnified. First, TNF-α-induced an approximately three-fold increase in the hypothalamic expression of the pro-apoptotic protein Bax (Fig. 3B), while in UCP2-inhibited rats, the effect of TNF-α was significantly increased, reaching an almost five-fold increase as compared to control and a 1.5-fold increase as compared to TNF-α treatment alone (Fig. 3B). Regarding the anti-apoptotic protein Bcl-2, TNF-α alone produced a significant reduction, leading to levels that corresponded to ~50% of control levels (Fig. 3C). The inhibition of UCP2 expression sig-

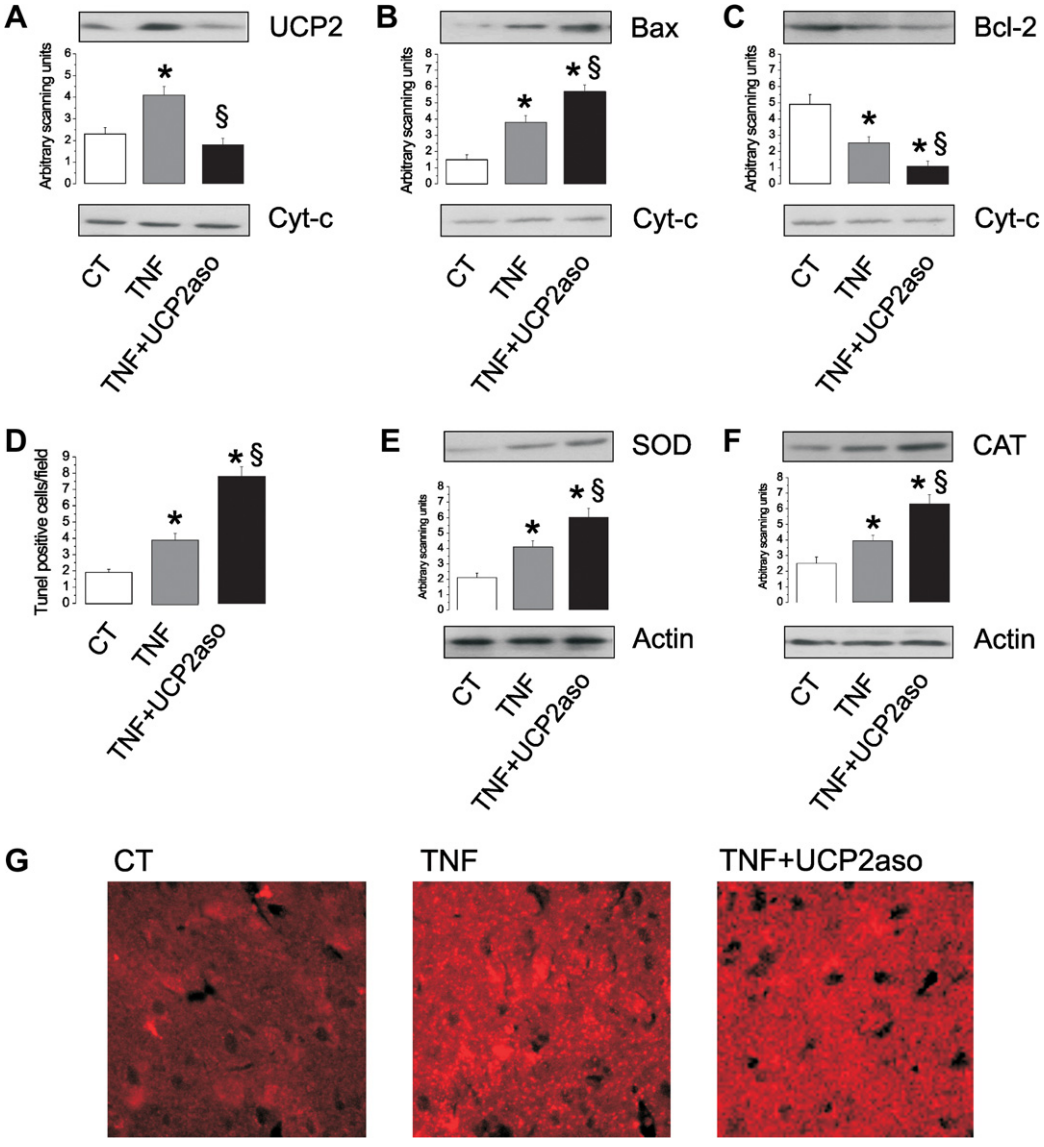

Fig. 3. Modulation of UCP2 (A), Bax (B) and Bcl-2 (C) expressions in hypothalamic mitochondria and SOD (E) and catalase (CAT) (F) in whole hypothalamus extract of untreated rats (CT), rats treated with TNF-α, or rats treated with TNF-α and previously exposed to UCP2 antisense oligonucleotides (TNF + UCP2aso). Samples were obtained from rats treated, ICV, either with saline (CT), TNF-α (10^{-12} M; single ICV injection), or TNF-α (10^{-12} M; single ICV injection) plus UCP2 anti-sense oligonucleotides during 9 days, (2.0 nmol/day, single IP injection/day), respectively. Cytochrome-*c* (Cyt-*c*) or actin were analyzed as loading controls. (D) Detection of apoptosis in hypothalamic cells by TUNEL. Analysis of TUNEL-positive staining (cells/field) in saline treated, TNF-α-treated rats and TNF-α-treated rats pre-exposed to UCP2 antisense oligonucleotides (UCP2 aso). (G) Fluorescence microscopy of 14 μm thick sections from hypothalamus (arcuate nucleus, Bregma −2.6) of rats treated with hydroethidin; ethidium was detected by excitation, 510 nm and emission 580 nm (magnification, 400×). Values are means ± S.E.M. (in A–C, E and F, $n = 4$; in D, $n = 5$; $^{*}P < 0.05$ vs. CT, $^{\S}P < 0.05$ vs. TNF); G, depicts typical acquisitions of $n = 3$.

nificantly potentiated the effect of TNF-α, producing a further 60% reduction in Bcl-2, as compared to TNF-α only. This corresponds to approximately 20% of the levels of Bcl-2 detected in the hypothalamic mitochondria of control rats (Fig. 3C). In parallel to the effects of UCP2 inhibition on Bax and Bcl-2, an effective modulation of apoptosis was detected. As shown in Fig. 3D, TUNEL-positive cells in the hypothalamus of TNF-α plus UCP2aso increased by more than two-fold, as compared to TNF-α-treated only rats, and by approximately four-fold, as compared to controls. Since apoptosis induced by pro-inflammatory cytokines can be mediated, at least in part, by ROS accumulation, we determined the expression of two important markers of ROS production, SOD and CAT. As shown in Fig. 3E and F, TNF-α alone significantly increased the expressions of both enzymes (2- and 1.5-fold for SOD and CAT, respectively), an effect that was significantly magnified by the simultaneous inhibition of UCP2 (1.5- and 1.7-fold for SOD and CAT, respectively). Moreover, TNF-α was also capable of inducing an increased hypothalamic accumulation of ethidium in hydroethidin treated rats, an effect that was further enhanced by the inhibition of UCP2 expression (Fig. 3G).

3.4. Cold-exposure simultaneously increases hypothalamic UCP2 expression and protects cells from TNF-α-induced apoptosis

In TNF-α-treated rats, the exposure to cold produced a 2.2-fold increase in hypothalamic UCP2 expression, as compared to controls, and a 1.4-fold increase, as compared to TNF-α treatment alone (Fig. 4A). This effect was accompanied by a significant reduction in Bax expression (~30% of TNF-α-treated only) to levels similar to those of the controls (Fig. 4B), and to a significant increase in Bcl-2 expression (1.5-fold, as compared to TNF-α-treated only) to levels similar to control, as well (Fig. 4C). The evaluation of apoptosis by TUNEL revealed that cold exposure was sufficient to completely inhibit TNF-α-induced apoptosis (Fig. 4D). Finally, cold exposure partially restored the levels of SOD and CAT, leading to levels ~40% and ~55% of TNF-α-treated only rats, respectively (Fig. 4E and F). However, the levels of the enzymes were still significantly higher than in controls (Fig. 4E and F).

3.5. Inhibition of UCP2 expression restrains the protective effects of cold exposure in markers of cell damage

To determine the role played by the increased expression of UCP2 in cold-exposed animals, regarding the damaging effects of TNF-α, a group of rats exposed to cold were treated with the antisense oligonucleotide to inhibit UCP2 expression and the expressions of Bax, SOD and CAT were evaluated. As shown in Fig. 5A, the treatment of cold-exposed/TNF-α-treated rats with UCP2 antisense oligonucleotide significantly reduced UCP2 expression to 45% of that seen in cold-exposed/TNF-α-treated only rats. Under UCP2 expression inhibition, a complete reversal of the protective effect of cold exposure upon Bax expression was obtained (Fig. 5B). Similarly, the levels of SOD (Fig. 5C) and CAT (Fig. 5D) were completely reversed to those of rats treated with TNF-α but not exposed to cold.

4. Discussion

Several cell types and tissues are known to be sensitive to prolonged inflammatory stimuli, undergoing apoptosis [18]. In the nervous system, apoptosis induced by continuous

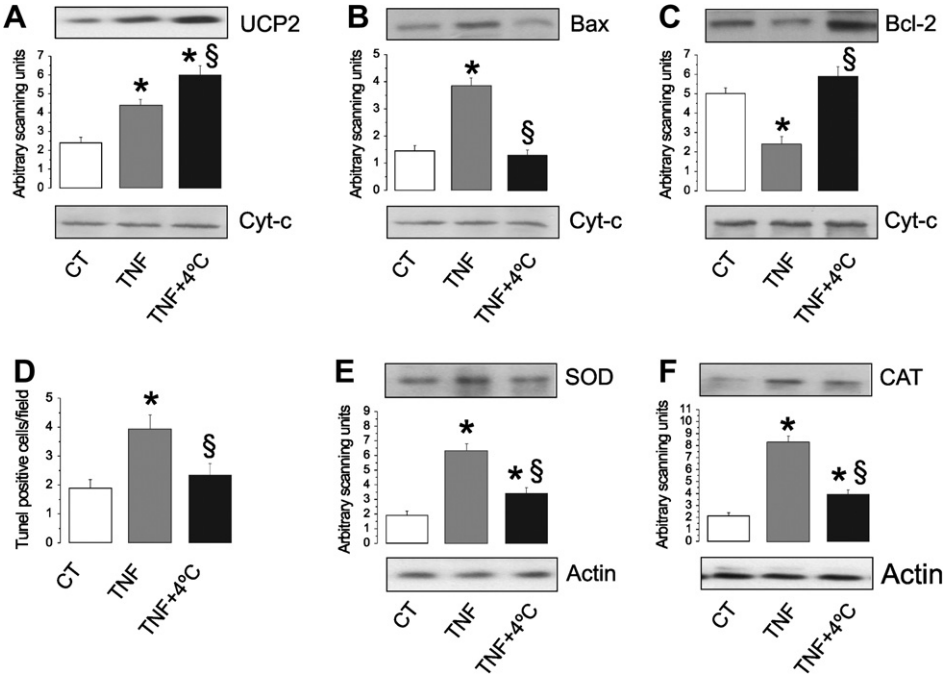

Fig. 4. Modulation of UCP2 (A), Bax (B) and Bcl-2 (C) expressions in hypothalamic mitochondria and SOD (E) and catalase (CAT) (F) in whole hypothalamus extract of untreated rats (CT), rats treated with TNF-α, or rats treated with TNF-α and exposed to cold (TNF + 4 °C). Samples were obtained from rats treated, ICV, either with saline (CT), TNF-α (10^{-12} M; single ICV injection), or TNF-α (10^{-12} M; single ICV injection) plus cold exposure for 9 days, respectively. Cytochrome-*c* (Cyt-*c*) or actin were analyzed as loading controls. (D) Detection of apoptosis in hypothalamic cells by TUNEL. Analysis of TUNEL-positive staining (cells/field) in saline treated, TNF-α-treated rats and TNF-α-treated rats plus cold exposure for 9 days. Values are means ± S.E.M. (in A–C, E and F, $n = 4$; in D, $n = 5$; $^{*}P < 0.05$ vs. CT, $^{\S}P < 0.05$ vs. TNF).

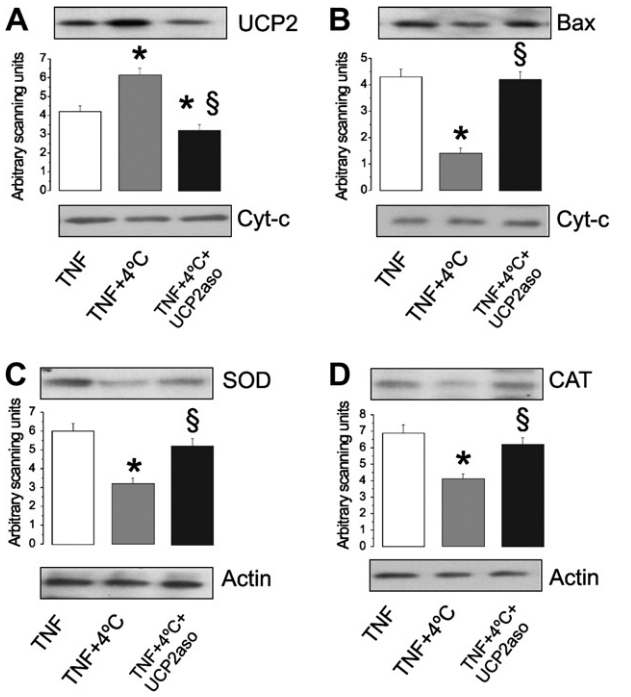

Fig. 5. Modulation of UCP2 (A) and Bax (B) expressions in hypothalamic mitochondria and SOD (C) and catalase (CAT) (D) in whole hypothalamus extracts of rats exposed either to TNF-α, TNF-α plus cold exposure (TNF + 4 °C), and TNF-α plus cold exposure in rats pre-treated with UCP2 antisense oligonucleotides (TNF + 4 °C + UCP2aso). Samples were collected from rats treated with TNF-α (10^{-12} M; single ICV injection), or TNF-α (10^{-12} M; single ICV injection) plus cold exposure for 9 days, or TNF-α (10^{-12} M; single ICV injection) plus cold exposure for 9 days plus UCP2 anti-sense oligonucleotides during the 9 days, (2.0 nmol/day, single IP injection/day); cytochrome-*c* (Cyt-*c*) or actin were used as loading controls. Values are means ± S.E.M ($n = 4$; $^{*}P < 0.05$ vs. TNF; $^{\S}P < 0.05$ vs. TNF + 4 °C).

inflammation plays a critical role in a number of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, epilepsy and amyotrophic lateral sclerosis [19,20]. Once installed, independently of its source, inflammation can disturb cellular homeostasis by multiple mechanisms [21]. Local production and action of inflammatory cytokines is regarded as one of the most important mechanisms linking inflammation to apoptosis [22,23]. The final fate of a given cell exposed to a pro-apoptotic stimulus depends not only on the type and magnitude of the stimulus, but also on the intrinsic capacity of the cell to resist the challenge. Recently, a number of studies have provided strong evidence to support a role for UCP2 as a protective factor against neuronal damage [4,24]. One of the proposed mechanisms for the effect of UCP2 is the control of ROS production [25]. Here, we tested the hypothesis that UCP2 acts as an endogenous protective factor against TNF-α-induced cell damage.

Initially, the capacity of TNF-α to induce apoptosis in the hypothalamus was tested. For this, ICV cannulated rats were treated for 6 days with a low dose of the cytokine and apoptosis was evaluated by two distinct methods. As previously demonstrated in a number of studies using both cells and intact tissues from several regions of the brain, in our hands, TNF-α produced an outstanding response, increasing the number of apoptotic cells in the hypothalamus [26,27].

Since our working hypothesis is that UCP2 expression would protect cells from TNF-α-induced damage, we next optimized the methods for detection of UCP2 in the hypothalamus. First, we evaluated the quality of the mitochondria preparation. For this a mitochondria respiration assay was settled and the effects of both TNF-α and cold exposure, reducing the respiratory rates of hypothalamic mitochondria and confirming the adequate experimental conditions, were determined. Next, during the optimization of the methods for detecting UCP2 expression, we observed that, although a protein band corresponding to UCP2 could be detected in whole tissue extracts, a considerably better result was obtained when working with proteins from isolated mitochondria. By employing this method, we established the optimal conditions for inhibiting UCP2 expression, using a previously described antisense oligonucleotide [11], and for increasing UCP2 expression, by exposing the experimental animals to a cold environment, as previously optimized [28].

To evaluate the role of UCP2 as a protective factor against TNF-α-induced damage in the hypothalamus, rats were treated simultaneously with TNF-α and with the antisense oligonucleotide against UCP2. First, we observed that ICV TNF-α alone induced a more than two-fold increase in UCP2 expression in the hypothalamus. Studies from the late 1990s explored the inflammatory mechanisms capable of inducing the expression of UCP proteins in several tissues. According to these studies, TNF-α is an important mediator of the effects of LPS towards UCP gene transcription, and proposed that this effect of TNF-α would be one of the mediators of inflammation and infection-induced fever [29]. When we used the antisense oligonucleotide, UCP2 protein was reduced to levels below those of the control. This fact resulted in increased expression of the pro-apoptotic protein, Bax, and reduced expression of the anti-apoptotic Bcl-2, and coincided with increased apoptosis in hypothalamus, as determined by the TUNEL method. This is the first evidence of a protective effect of UCP2 in TNF-α-induced apoptosis in nervous tissue. It may explain for example, some of the protective effects of UCP2 in stroke and trauma and provides further support for the proposed anti-apoptotic effects of UCP2 in peripheral tissues [30,31].

Since the inhibition of ROS production is believed to be one of the most important mechanisms involved in UCP2-dependent neuronal protection, we evaluated the impact of UCP2 inhibition on TNF-α-induced SOD and CAT expression and on ethidium accumulation following hydroethidin treatment. TNF-α alone produced an approximately two-fold increase in SOD and CAT expression in the hypothalamus. In isolated neural cells [32] and in peripheral cells and tissues, TNF-α has been exhaustively shown to stimulate the expression of these enzymes. When UCP2 was inhibited, the expressions of both enzymes were significantly increased, strongly suggesting that at least part of the damaging effect of reduced UCP2 expression was linked to increased ROS production. Indeed, when employing a method that indirectly estimates O^{2-} and O^{2-} derived oxidant production by determining the local accumulation of ethidium, an oxidized product of hydroethidin, similar results were found.

As an indirect means of evaluating the effect of increased UCP2 expression in TNF-α-induced damage of hypothalamic cells, rats were exposed to cold and then treated with the cytokine. The exposure to cold alone promoted a significant in-

crease in UCP2 expression and, when comparing TNF-α treatment alone with TNF-α plus cold exposure, the difference in UCP2 protein level was about 1.4-fold. In parallel to the effect of cold on UCP2 expression, a remarkable inhibition of Bax and a stimulation of Bcl2 plus a significant inhibition of apoptosis was observed. All these effects were accompanied by a reduction in the expression of the enzymes SOD and CAT. Therefore, cold exposure is capable of significantly reducing the damaging effects of TNF-α in cells of the hypothalamus. The ability of cold exposure to induce the expression of UCP2 in neural tissue is already known [33]; however, this is the first report of a synergic effect of a cytokine and cold exposure to increase the expression of UCP2. Cold exposure can modulate the expression of a number of different proteins in the hypothalamus, as shown in some recently published studies [34–36]. Therefore, it could be argued that the effects of cold described herein may not be dependent on UCP2 regulation. To provide further support to our hypothesis, we inhibited UCP2 expression in cold-exposed/TNF-α-treated rats. This approach led to a complete reversal of the beneficial effects of cold exposure on Bax, SOD and CAT.

In conclusion, the induction of UCP2 in the hypothalamus of rats is an endogenous protective mechanism that minimizes the harmful effects of a potent inflammatory stimulus. The modulation of UCP2 expression or activity may result in an interesting therapeutic approach for inflammatory diseases of the central nervous system.

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